Type II Collagen Accumulation in Overlying Dermo-Epidermal Junction of Pilomatricoma Is Mediated by Bone Morphogenetic Protein 2 and 4

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Pilomatricoma consists of the cells differentiating towards hair matrix cells. Immunohistochemical study revealed the deposition of type II collagen in the overlying dermo-epidermal junction (DEJ) of this benign tumor. Proα_1(II) mRNA was detected by RT-PCR in the overlying epidermal layer but not in the dermal layer prepared from the lesional skin of pilomatricoma. The neutral salt-soluble proteins extracted from the tumor of pilomatricoma induced proα_1(II) mRNA in the cultured human keratinocytes but not in the cultured dermal fibroblasts. Bone morphogenetic protein 2 or 4 (BMP2 or 4) was immunohistochemically detected in some shadow cells of pilomatricoma. Recombinant BMP2 and BMP4 were found to induce proα_1(II) mRNA concentration dependently in the cultured human keratinocytes but not in the cultured fibroblasts. Proα_1(II) mRNA induced by BMP2 and in cultured keratinocytes contained exon 2, indicating that the mRNA species is non-chondrogenic type IIA form. The results strongly suggest that BMP2 or 4 expressed in pilomatricoma is responsible for the induction of proα_1(II) collagen mRNA in the overlying epidermal cells resulting in the deposition of type II collagen in the DEJ. When human keratinocytes were cultured on type II collagen substratum *in vitro*, the cell proliferation was accelerated at the early period of culture but was inhibited at the late period of culture, whereas the cell proliferation was persistently accelerated by type I or IV collagen substratum. Type II collagen deposition in the DEJ may potentially exert profound effects on keratinocyte proliferation and differentiation.

Key words: bone morphogenic protein 2 and 4/hair follicle/keratinocyte differentiation/pilomatricoma/type II collagen

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Pilomatricoma or calcifying epithelioma of Malherbe is a benign cutaneous epithelial tumor, and morphologically composed of small basophilic cells and large, probably non-viable shadow cells as well as transitional cells between basophilic and shadow cells (Forbis and Helwig, 1961). Previous immunohistochemical and histochemical studies demonstrated that majority of pilomatricoma cells are considered to be differentiating cells towards hair-forming cells, particularly hair cortex cells, of hair follicles (Moll *et al*, 1988; Watanabe *et al*, 1994). The mechanism of epidermal cell differentiation towards hair matrix cells remains to be determined. Recently β-catenin, a participant in the Wnt signaling, has been shown to play an important role in the morphogenesis of hair follicles and the tumorigenesis of fair follicle-related tumors including pilomatricomas. β-catenin is essential for fate decisions of stem cells between the formation of follicular and epidermal keratinocyte lineages (Gat *et al*, 1998; Huelsken *et al*, 2001), and 26% or 75% of pilomatricomas possess activating mutations in exon 3 of β-catenin gene (Chan *et al*, 1999; Moreno-Bueno *et al*, 2001).

Bone morphogenetic proteins (BMPs) are considered to be members of transforming growth factor β superfamily based on their amino acid sequence homology. BMPs exhibit chondrogenic and osteogenic properties *in vivo* and *in vitro* (Wozney *et al*, 1988). For examples, implantation of recombinant human BMP in rat induces bone formation (Wang *et al*, 1990). Incubation of bone marrow-derived mesenchymal progenitor cells with BMP induces chondrogenic differentiation *in vitro* (Johnstone *et al*, 1998; Sekiya *et al*, 2001). Besides the chondrogenic or osteogenic activity, BMP2 and BMP4 are potential hair follicle initiation factors and regulators of hair cycle (Blessing *et al*, 1993; Botchkarev, 2003). Increased evidences suggest that the expression of BMP depends on β-catenin during follicular formations and hair cycling, although whether BMP is a direct or indirect target gene of β-catenin signaling in hair formation is currently unknown (Huelsken *et al*, 2001).

Type II collagen is the major and unique collagenous component of cartilage and plays a critical role in chondrogenesis during embryonic development (Mayne and von der Mark, 1983). It has been demonstrated that

Abbreviations: DEJ, dermo-epidermal junction; EDTA, ethylenediaminetetraacetic acid; Ig, immunoglobulin; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecylsulfate; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate

type II collagen is much more widely distributed during early development than previously thought. Type II collagen is detected in non-chondrogenic tissues including embryonic notochord (Linsenmayer *et al*, 1973; Miller, 1974), corneal epithelium (Linsenmayer *et al*, 1977), neural retina (Smith *et al*, 1976), ectodermal-mesenchymal interfaces throughout the trunk of the chick embryo at the very early stages (stages 14–19; days 2–3) (Thorogood *et al*, 1986; Kosher and Solursh, 1989) and the subepidermal matrix of mouse hindlimb (stage 31; 7.5 d) which subsequently disappears with development (Fitch *et al*, 1989, 1990). We have previously demonstrated that type II collagen is detected in the dermo-epidermal junction (DEJ) excluding the region at which the hair follicles are forming in the human fetal scalp skin at the stages of 17–23 fetal weeks (Azuma *et al*, 1994), implying that type II collagen expression in the DEJ precedes the formation of hair follicles in the fetal skin development.

In the course of the studies exploring the expression of type II collagen in the postnatal skin, we found the expression of type II collagen in the overlying DEJ of pilomatricoma and demonstrated that the induction was mediated by BMP2 or BMP4 detected in the tumor cells. We further studied the influence of type II collagen deposition on keratinocyte growth and differentiation in the cell culture system. The results in this study indicate that keratinocytes in the postnatal skin is still pluripotential and can express type II collagen under the pathological condition.

Results

Detection of type II collagen in overlying DEJ of pilomatricoma and localization of proα_1(II) mRNA Immunohistochemical study of pilomatricoma using anti-type II collagen antibody demonstrated that *four* cases of pilomatricoma exhibited a strong staining in the overlying DEJ. The immunoreactivity was restricted in DEJ, and no apparent immunoreactivity was found in the dermal matrices (Fig 1*a* and *b*). Normal skins obtained from benign skin tumors including epidermal cyst ($n = 5$) and nevus cell nevus ($n = 5$) showed negative reaction with this antibody (Fig 1*c*).

Localization of proα_1(II) mRNA was studied by RT-PCR using the RNA prepared from the lesional epidermis and dermis of cases 1 and 2 pilomatricomas. Specific 369 bp fragment of proα_1(II) mRNA was found to be detected preferentially in the overlying epidermis of pilomatricoma not in the dermis in both cases (data not shown).

Detection of BMP2 or BMP4 in pilomatricoma Paraffin-embedded sections were incubated with anti-BMP2/4 antibody. BMP2/4 was detected in some shadow cells of pilomatricoma in both cases 1 and 2 but not in the basophilic or transitional cells (Fig 2), which was consistent with the previous report (Kurokawa *et al*, 2000). There was no immunoreactivity of BMP2/4 in the overlying epidermal cells and dermal cells (not shown).

BMP2 and BMP4 induce type II collagen expression in cultured human keratinocytes We first speculated that some soluble factor(s) may be secreted from the tumor and act on basal keratinocytes to induce the expression of type II collagen. In order to prove this, tumor mass of pilomatricoma was homogenized and sonicated in the neutral buffer (0.1 M Tris-HCl, pH 7.5, containing protease inhibitor cocktail). Human keratinocytes and fibroblasts were treated with the homogenate of pilomatricoma at the dose of 10 μg per mL medium for 24 h. RT-PCR clearly showed that the homogenate induced 369 bp fragment of proα_1(II) mRNA in the cultured keratinocytes but not in the cultured fibroblasts (Fig 3*a*). To identify the factor(s) in the homogenate, ELISA was done using the antibodies for various factors including tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β), and interleukin-1β (IL-1β). But these factors gave negative results (not shown). Because the result of immunohistochemistry of shadow cells of piloatricoma with the antibody for BMP2/4 strongly suggested that the candidate factors were BMP2 and

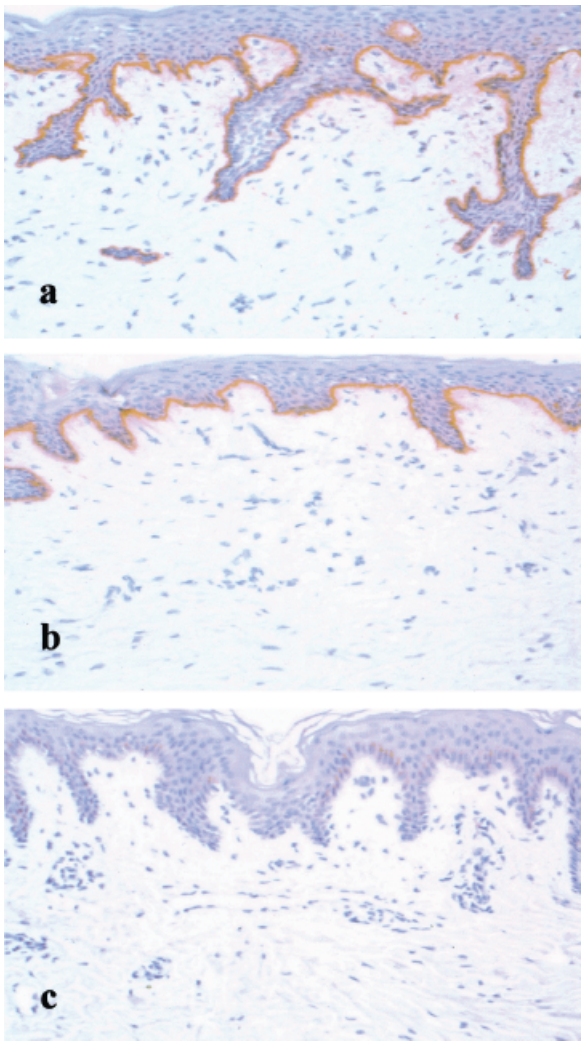

Figure 1
Immunohistochemical staining of the overlying skin of pilomatricoma with monospecific anti-type II collagen antibody. Overlying skins of case 1 (*a*) and case 2 (*b*) of pilomatricoma and normal skin (*c*) were incubated with anti-type II collagen antibody at 1:40 dilution, then with biotin-conjugated anti-mouse immunoglobulin antibody. Localization of antigen was visualized with avidin–biotin complex (original magnification $\times$ 100).

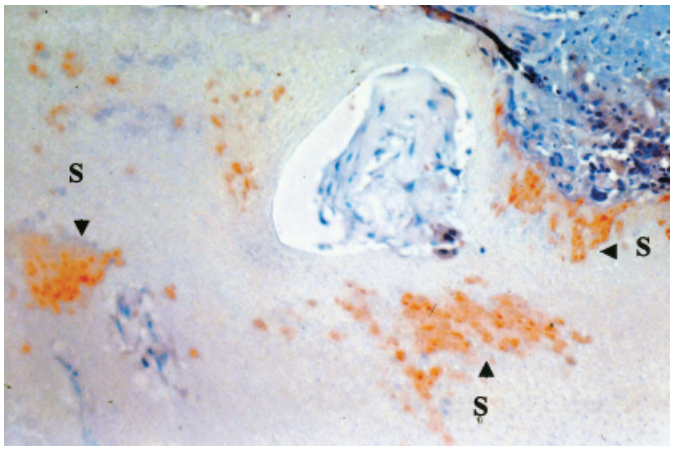

Figure 2
Immunohistochemical detection of bone morphogenetic protein 2 or 4 (BMP2 or BMP4) in pilomatricoma. Skin specimens obtained from cases 1 and 2 were incubated with anti-BMP2/4 antibody for 24 h at 4°C, then incubated with biotin-conjugated anti-goat Ig antibody at 1:500 dilution for 2 h at room temperature. Antigen–antibody complex was reacted with avidin–biotin complex. Original magnification is × 100. S, shadow cell.

BMP4, we continued the further study focusing on BMP2 and BMP4.

To prove the type II collagen-inducible activity of BMP2 and BMP4 directly, we studied whether proα_1(II) mRNA is induced in the cultured human keratinocytes by the treatment of various amount of BMP2 or BMP4. RT-PCR demonstrated that specific 369 bp fragment of proα_1(II) transcript was detected after the treatment of BMP2 and BMP4 at the concentrations higher than 100 ng per mL (Fig 3*b*). By contrast, dermal fibroblasts did not induce proα_1(II) transcript by BMP2 and BMP4 (not shown) at the concentrations tested here. Pre-treatment of the cells with recombinant human noggin (1.6 ng per mL) inhibited BMP2-inducible proα_1(II) transcript enhancement (Fig 3*c*).

Structual analysis of proα_1(II) mRNA induced in overlying epidermis and cultured keratinocyte Since it has been demonstrated that type II procollagen is expressed in two structurally different mRNAs by alternative splicing of the primary gene transcript that either include (type IIA) or exclude (type IIB) an exon 2 encoding the major portion of the amino terminal propeptide (Ryan and Sandell, 1990; Sandell *et al*, 1991), we performed the structural analysis of proα_1(II) mRNA induced by BMP2 and BMP4 in the cultured keratinocytes. One single fragment of 377 bp in both BMP2- and BMP4-treated keratinocytes was detected by RT-PCR (Fig 4*a*, lanes 2 and 4), whereas 171 bp fragment was detected in the auricular cartilage (Fig 4*a*, lane 5), indicating that proα_1(II) mRNA species induced by BMP2 and BMP4 is type IIA form and those of auricular cartilage is type IIB form. To confirm this result, RT-PCR products were blotted onto the filters and hybridized with the oligonucleotide probe spanning 3′ end of exon 1 and 5′ end of exon 2. A 377 bp fragment was detected by autoradiography in both BMP2- and BMP4-treated keratinocytes but 171 bp fragment in the ear cartilage was not (Fig 4*b*), indicating that proα_1(II) mRNA species was type IIA form.

Type II collagen substratum inhibits keratinocyte proliferation in late phase of culture In order to investigate the biological significance of type II collagen accumulated in the DEJ, effect of type II collagen on keratinocyte proliferation was studied *in vitro*. Cells were plated at a relatively low density of 1×10^3 per 32 mm diameter dishes to observe the change of cell growth for longer periods. Type II collagen substratum as well as types I and IV collagen substrata showed a growth-promoting effect at the early phase in culture (days 4 and 6 in culture) but type II collagen substratum exhibited a significant inhibiting effect at the late phase of culture (days 12 and 14 in culture) ($p < 0.01$) when type I collagen and type IV collagen still showed growth-stimulating effect (Fig 5). A considerable number of cells on

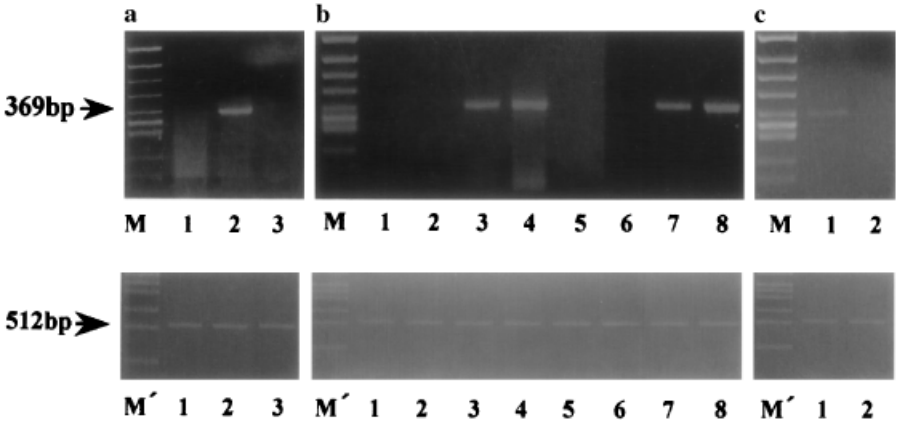

Figure 3
Proα_1(II) collagen transcript is induced in cultured human keratinocytes by the homogenate prepared from pilomatricoma, or by recombinant human bone morphogenetic protein 2 and 4 (BMP2 and BMP4). Cultured keratinocytes and fibroblasts were treated with the homogenate prepared from pilomatricoma (*a*), or cultured keratinocytes were treated with recombinant BMP2 and BMP4 (*b*), or treated with the combination of recombinant BMP2 and recombinant noggin (*c*). RNA was isolated from the cultured keratinocytes. RT-PCR was performed using proα_1(II) (upper panel) and β-actin (lower panel) primer pairs. PCR products were analyzed by an electrophoresis on agarose gel. (*a*) Keratinocytes were treated for 24 h without (lane 1) or with (lane 2) the homogenate isolated from pilomatricoma. Fibroblasts were treated for 24 h with the homogenate (lane 3). (*b*) Keratinocytes were treated for 24 h with 0 (lane 1), 25 (lane 2), 100 (lane 3), and 250 ng per mL (lane 4) BMP2, or treated with 0 (lane 5), 25(lane 6), 100 (lane 7), and 250 (lane 8) ng per mL BMP4. (*c*) Keratinocytes were treated for 24 h with 100 ng per mL BMP2 alone (lane 1) or with the combination of 100 ng per mL BMP2 and 1.6 ng per mL noggin (lane2). M indicates molecular markers of 770, 612, 495, 392, 341, 297, 210 bp. M′ indicates molecular markers of 1360, 1107, 926, 658, 489, 267 bp.

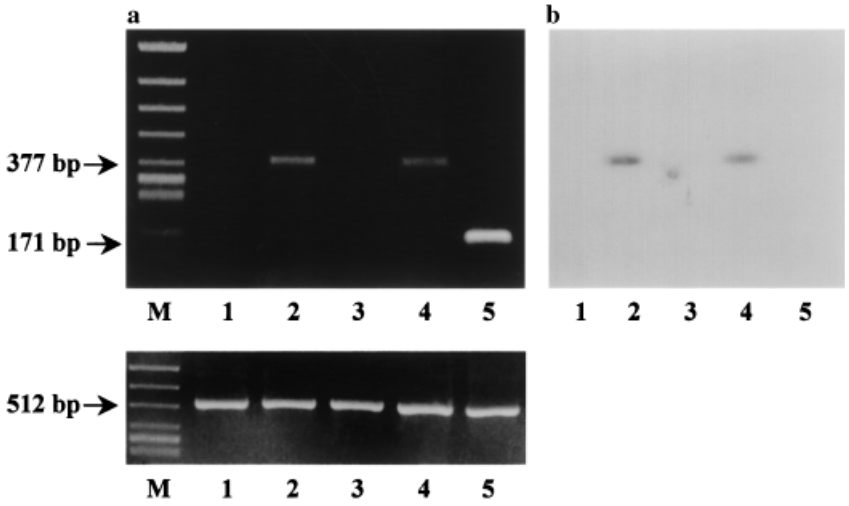

Figure 4
Alternative splicing of exon 2 of type II collagen primary transcript expressed by cultured keratinocytes in the presence of bone morphogenetic protein 2 or 4 (BMP2 or BMP4). (*a*) Keratinocytes were treated without (lanes 1 and 3) or with either BMP2 (250 ng per mL) (lane 2) or BMP4 (250 ng per mL) (lane 4) for 48 h. RNA was isolated and RT-PCR was performed using upstream (exon 1) and downstream (exon 5) primers of proα_1(II) (upper panel) and β-actin (lower panel). PCR product was analyzed by agarose gel electrophosesis. Positive control assays were done using the RNA isolated from ear cartilage (lane 5). M indicates molecular markers of 770, 612, 495, 392, 341, 297, 210 bp. (*b*) RT-PCR products were blotted onto filters and hybridized with the radiolabeled oligonucleotide probe spanning 3′ end of exon 1 and 5′ end of exon 2. The filters were washed and subjected to autoradiography.

type II collagen substratum on days 12 and 14 was found to float in the medium under a microscope (not shown).

Discussion

We have found type II collagen deposition in overlying DEJ of pilomatricoma. Overlying epidermal keratinocytes will be responsible for the expression of type II collagen because type II collagen mRNA was detected in the overlying epidermis not in the dermal tissue, and type II collagen mRNA was induced by BMP2 and BMP4 treatments in the cultured keratinocytes but not in the cultured fibroblasts. This is an interesting observation because extracellular matrix proteins in DEJ including type IV collagen are considered to be produced through epidermal–dermal interaction (Fleischmajer *et al*, 1993; Marinkovich *et al*, 1993). In addition, transient expression of type II collagen in the ectodermal–mesenchymal interfaces during the early stage of chick embryonic development is considered to be mediated by epithelial–mesenchymal interaction (Thorogood *et al*, 1986; Kosher and Solursh, 1989).

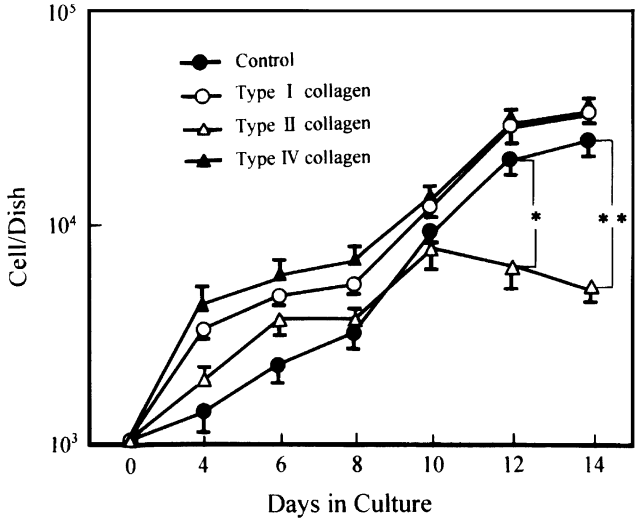

Figure 5
Cell proliferation of cultured keratinocytes is suppressed in type II collagen-coated dishes. Human keratinocytes were plated at an intial density of 1×10^3 per dish on type I(open circle), type II (open triangle), type IV collagen-coated (closed triangle) or plain (closed circle) dishes and maintained in KGM over 14 d. Cell number was counted every 2 d after trypsin treatment. Values are mean $\pm$ SEM from two sets of assays done in triplicate. Statistical significance at $p < 0.01$ is indicated by * and **.

We have found that the homogenate extracted from pilomatricoma by neutral salt is capable of inducing type II collagen expression in cultured keratinocytes. In the course of searching the candidate growth factor or cytokines responsible for the type II collagen induction, we found BMP2/4 expression in the shadow cells of pilomatricoma but not in the other cell components of pilomatricoma. On the basis of the hypothesis that BMP2 and BMP4 are potent inducible factor of type II collagen, we treated the cultured keratinocytes with BMP2 and BMP4 and found their type II collagen-inducing activity. This was confirmed by the experiment that the induction of type II collagen by BMP2 was abolished in the presence of noggin, a specific agonist of BMP2 and BMP4. We have tested some other cytokines and growth factor such as TGF-β1, TNF-α, or IL-1β but failed to find such activities (not shown). This suggests that BMP2 and BMP4 produced and secreted by shadow cells of pilomatricoma act on overlying epidermis to induce type II collagen. In fact, in the normal-appearing skin external from pilomatricoma type II collagen deposition in DEJ was not be able to be detected (not shown). These results are consistent with the previous studies that the expression of BMPs have an essential role in cutaneous tumorigenesis (Blessing *et al*, 1995, 1996; Wach *et al*, 2001).

The question whether type II expression in DEJ is the cause or result of pilomatricoma is still unclear. We initially thought that some factors involving in the differentiation of stem cells towards hair cortex cells in the tumorigenesis of pilomatricoma are also involved in the induction of type II collagen expression in the DEJ. But the presence of detectable BMP2/4 in the shadow cells of pilomatricoma and type II collagen-inducing potential of BMP2 and BMP4 in cultured keratinocytes may suggest that pilomatricoma itself potentially expresses BMP2/4 which induces the expression of type II collagen in the DEJ. Therefore we at present think that type II collagen expression is the result rather than cause of pilomatricoma.

Type II collagen has been reported to be expressed in two mRNAs by differential splicing of the primary gene transcript that either includes (type IIA) or excludes (type IIB) an exon 2 encoding the major portion (69 amino acids) of the amino terminal propeptide (Ryan and Sandell, 1990). Each procollagen mRNA has a distinct tissue distribution during chondrogenesis, with type IIB expressed in chondrocyte and type IIA expressed in the chondroprogenitor cells surrounding the cartilage (Sandell *et al*, 1991). Proα_1(II) mRNA induced by BMP2 and BMP4 was found to contain exon 2 (type IIA), indicating that type II collagen in DEJ of pilomatricoma is non-chondrogenic from (type IIA).

Physiological significance of type II collagen in the DEJ is uncertain at present. Accumulated type II collagen in the DEJ may exert a profound influence on the stability of DEJ as well as the differentiation of keratinocytes and the structural organization of adjacent extracellular matrix components (such as anchoring fibrils) in the subepidermal region since in the normal condition type II collagen never exists in this area except a very short duration of chick embryonic and human fetal skin development (Azuma *et al*, 1994). Transient expression of type II collagen in chick embryonic and human fetal scalp skins is thought to be related to the development of feather and hair follicle, because this collagen starts to diminish at the restricted sites where feather buds and hair follicles are being developed. If type II collagen induction in the overlying DEJ of pilomatricoma occurs by the same mechanism as seen in the chick embryonic and human fetal skin development, type II collagen in pilomatricoma induced by BMP2/4, which are potent hair follicle initiation factors (Blessing *et al*, 1993), will be a preceding marker protein of the induction of hair follicles. Keratinocyte differentiation to hair matrix occurs in the lesions other than pilomatricomas such as follicular cysts and cutaneous mixed tumors (LeBoit *et al*, 1987). We found type II collagen accumulation in the overlying DEJ of some cases of trichilemmoma and cutaneous mixed tumor as well (manuscript in preparation). This will also support the idea that type II collagen expression in the DEJ is related to follicular differentiation of keratinocytes.

Previous experiments have shown that extracellular matrices like types I and IV collagens, fibronectin, and laminin influence keratinocyte adhesion, spreading, proliferation, and differentiation (Kubo *et al*, 1987; Adams and Watt, 1989; Guo and Grinnell, 1989; Woodley *et al*, 1990). In fact, type I or IV-, fibronectin-, and laminin-coated plastic dishes have been used to efficiently maintain keratinocyte growth in culture. The interaction between keratinocytes and type II collagen has never been studied because expression of type II collagen has never been expected in DEJ or subepidermal area of the skin. It is noted that type II collagen, like other extracellular matrices, promoted keratinocyte proliferation at the early stage of culture, but unlike other extracellular matrices, inhibited keratinocyte proliferation. It is uncertain that the biphasic effect of type II collagen substratum on keratinocyte proliferation is due to the difference of cell density during the cell proliferation or due to the switching of cell signaling pathway mediated by the interaction between keratinocyte and type II collagen. The histological changes in the overlying epidermis of pilomatricoma such as the formation of horn cyst, proliferation of basalioma-like cells (Aso *et al*, 1990), or abnormal elongation of epidermal rete ridge (histologically seen in case 1 in Fig 1) may be related to the altered keratinocyte differentiation induced by the accumulation of type II collagen in the DEJ.

Materials and Methods

Skin samples Skin samples were obtained from *four* cases of pilomatricoma under the permission of the patients and subjected to histological and biological analysis. Case 1: 22-y-old Japanese woman noticed skin nodule on the right arm for 1 y. Physical examination showed 21 $\times$ 25 mm intradermal tumor with clear border. Case 2: 15-y-old Japanese girl presented with asymptomatic dermal tumor with the diameter of 12 mm on the neck. She noticed the tumor 3 y before. Case 3: 17-y-old Japanese boy presented with asymptomatic dermal tumor with the diameter of 15 mm on the back. He noticed the tumor 3 y before. Case 4: 16-y-old Japanese girl presented with asymptomatic dermal tumor with the diameter of 30 $\times$ 25 mm on the back. She noticed the tumor 2 y before. The skin tumors were resected by a surgical operation. Histological diagnosis of pilomatricoma was done with hematoxylin–eosin stain. Normal skins (n = 10) were obtained from normal-appearing area of benign skin tumors (epidermal cyst and nevus cell nevus).

Immunohistochemistry Skin samples were fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μm sections. The sections were pre-treated with pronase (0.1% in phosphate-buffered saline) (type XX TV, Sigma, St Louis, Missouri) for 30 min, then incubated for 24 h at 4°C with monoclonal anti-human type II collagen antibody (Fuji Chemical Industries, Toyama, Japan) at 1:40 dilution, anti-human polyclonal BMP2/4 antibody (Genzyme/Techne, Minneapolis, Minnesota) at 1:100 dilution. The sections were incubated with biotin-conjugated anti-mouse immunoglobulin (Ig) antibody or anti-goat Ig antibody (Dako, Glostrup, Denmark) at 1:500 dilution for 2 h at room temperature. Antigen–antibody complex was reacted with peroxidase-labeled avidin–biotin complex (Dako) for 30 min. The reaction was visualized with 3-amino-9-ethylcarbazole. The sections were counterstained with hematoxylin. The specificity of anti-type II collagen antibody has been previously demonstrated by western blot assay (Azuma *et al*, 1994) and immunohistochemical studies using the specimen of human auricular cartilage (not shown).

Cell culture and treatment Normal human keratinocytes (NHKs) were purchased from Sanko-Junyaku (Tokyo, Japan) and cultured in serum-free, low-calcium (0.09 mM), modified MCDB 153 keratinocyte basal media containing the following growth factors, insulin, epidermal growth factor (EGF), hydrocortisone and bovine pituitary extract (BPE) (designated as KGM). NHKs at third or fourth culture were used in this study according to supplier's recommendation. Normal human skin fibroblast culture was established by explant method. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were plated at a density of 1×10^4 in 35 mm diameter dishes and grown for 6 d. Tumor mass ($\sim$ 1 g wet weight) was homogenized in 2 mL of 50 mM Tris-HCl, pH 7.4, containing protease inhibitor cocktail (1 mM EDTA, NEM, and PMSF) with a dounce homogenizer, and sonicated with five 30 s bursts (Handy Sonic UR-20P, Tomy Seiko Co., Tokyo, Japan) at 4°C. The homogenate was cleared by centrifugation at 15,000 g for 30 min at 4°C. The protein content of the supernatant was measure by absorbance at 280 Å. Cells were treated with the homogenate (final concentration; 10 μg per mL medium) for 24 h or human recombinant BMP2 and BMP4 (Pharma Biotechnology, Hannover, Germany) for 24 h at the doses of 0–250 ng per mL. To study the BMP-specific induction of type II collagen expression, cells were

treated for 24 h with the combination of BMP2 (100 ng per mL) and recombinant human noggin (1.6 ng per mL) (Pepro Tech EC Ltd, London, UK), a potent agonist for BMP2 and BMP4 (Zimmerman, 1996).

To study the effect of type II collagen on keratinocyte proliferation, cells were plated at a density of 1×10^3 per 35 mm diameter matrix-coated dishes containing either type I collagen from rat tail tendon, type II collagen from bovine cartilage or type IV collagen from human placenta (Beckton-Dickinson Labware, Bedford, MA). The matrix-coated dishes were prepared by the incubation of tissue culture dishes with these matrix proteins at the concentration of 10 μg per mL for 24 h at 4°C, followed by rinse with KGM twice before use (Kubo *et al*, 1987). Cells were grown in KGM for 14 d. On days 4, 6, 8, 10, 12, and 14 in culture, the cells were trypsinized and cell number was counted with a Coulter counter. Assays were performed twice in triplicate. Values were expressed by mean ± SEM. Statistical significance was calculated by one-sided Student's *t* test and p-value below 0.05 was considered to be significant.

RNA extraction and RT-PCR Total cellular RNA was isolated from the cultured keratinocytes or dermal fibroblasts with guanidine thiocyanate (Chomczynski and Sacchi, 1987), and adjusted to a concentration of 1 μg per μL. In an experiment, to separate the epidermis from dermis, the lesional skins of the pilomatricomas were cut into small pieces (~ 1 cm^3) and treated with 0.5% dispase (Sanko-Junyaku) at 4°C for 2 h. Epidermal layer was peeled off from the dermal layer by sterile forceps, then RNA was immediately isolated from both layers. For a control experiment, RNA was prepared from auricular cartilage obtained from surgical operation of squamous cell carcinoma on the ear. The exon-specific primer pairs; 5′ACACTCAAGTCCCTCAACAACCAGAT3′ for upstream primer and 5′GACGTCCAGATGACCTTCCTGC-GCCTG3′ for downstream primer at the C-terminal propeptide domain of human proα_1(II) chain (Sangiorgi *et al*, 1985) were synthesized. For internal standard, the upstream primer 5′TTAATGTCACGCACGATTTCCC3′ and the downstream primer 5′GTGATGGTGGCATGGGTCA3′ of β-actin cDNA (Ponte *et al*, 1984) were also synthesized. Total RNA (1 μg) was used for first strand cDNA synthesis. RT-PCR was performed in the presence of 3 pmol of a 3′-oligonucleotide and 35 U of reverse transcriptase from avian myoblastosis virus (Takara Shuzo Co., Otsu, Shiga, Japan) in a total reaction volume of 20 μL (Kawasaki, 1990). The resulting cRNA was then subjected to the first PCR using 25 pmol 5′-oligonucleotide and additional 3′-oligonucleotide in a total volume of 100 μL. The cDNA was amplified for 35 cycles at 94°C per 1 min, 58°C per 1 min, and 72°C per 2 min. Analysis of all the PCR products was performed by agarose (1.5%) gel electrophoresis followed by ethidium bromide staining. The PCR products were subcloned into a TA cloning vector (Invitrogen Corp., San Diago, California). Plasmid DNA was isolated (Wizard plus Miniprep, Promega Corp., Madison, Wisconsin), and subjected to nucleotide sequencing by dideoxy chain termination method (Sanger *et al*, 1977) to identify the cloned cDNA.

Structural analysis of proα_1(II) mRNA To analyze the splicing of exon 2 of proα_1(II) mRNA, the upstream primer 5′ATGATTCG3′ corresponding to the exon 1 sequences of proα_1(II) cDNA and downstream primer 5′AGGCCCAGGAGGTCCTTTGGG3′ corresponding to exon 5 sequences were synthesized (Sangiorgi *et al*, 1985). RNA was isolated from cultured keratinocytes which had been treated with BMP2 and BMP4. RT-PCR was performed as described above. Result of RT-PCR is expected to give PCR product with 377 bp in type IIA mRNA and 171 bp in type IIB mRNA.

To confirm the expression of proα_1(II) mRNA species in which exons 1 and 2 are contiguous, a 24 bp oligonucleotide probe (5′TGCCAGCCTCCTGGACATCCTGGC3′) corresponding to 12 nucleotides at 3′ end of exon 1 and 12 nucleotides at the 5′ end of exon 2 was synthesized as previously described (Ryan and Sandell, 1990), then radioactively labeled at 5′ end with [γ-^{32}P]ATP (210 TBq per mmol, Amersham, Biosciences Corp., Piscataway, New Jersey) and T4 polynucleotide kinase. RT-PCR products which had been resolved on 1.5% agarose gel were capillary-transferred onto nylon filters, then hybridized at 27°C overnight with the exons 1–2-specific oligonucleotide probe in a solution of 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10% dextran sulfate, 1% SDS, and 100 μg per mL tRNA. The filters were washed twice with $1 \times$ SSC at 55°C for 2 h, then subjected to autoradiography.

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